

Redundancy and Specificity of *Escherichia coli* Iron Acquisition Systems during Urinary Tract Infection[∇]

Erin C. Garcia, Ariel R. Brumbaugh, and Harry L. T. Mobley*

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109

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Uropathogenic *Escherichia coli* (UPEC), the predominant cause of uncomplicated urinary tract infection (UTI), utilizes an array of outer membrane iron receptors to facilitate siderophore and heme import from within the iron-limited urinary tract. While these systems are required for UPEC *in vivo* fitness and are assumed to be functionally redundant, the relative contributions of specific receptors to pathogenesis are unknown. To delineate the relative roles of distinct UPEC iron acquisition systems in UTI, isogenic mutants in UPEC strain CFT073 or 536 lacking individual receptors were competed against one another *in vivo* in a series of mixed infections. When combinations of up to four mutants were coinoculated using a CBA/J mouse model of ascending UTI, catecholate receptor mutants (Δ *fepA*, Δ *iha*, and Δ *iroN* mutants) were equally fit, suggesting redundant function. However, noncatecholate siderophore receptor mutants, including the Δ *iutA* aerobactin receptor mutant and the Δ *fyuA* yersiniabactin receptor mutant, were frequently outcompeted by coinoculated mutants, indicating that these systems contribute more significantly to UPEC iron acquisition *in vivo*. A tissue-specific preference for heme acquisition was also observed, as a heme uptake-deficient Δ *hma* Δ *chuA* double mutant was outcompeted by siderophore receptor mutants specifically during kidney colonization. The relative contribution of each receptor to UTI only partially correlated with *in vivo* levels of receptor gene expression, indicating that other factors likely contributed to the observed fitness differences. Overall, our results suggest that UPEC iron receptors provide both functional redundancy and niche specificity for this pathogen as it colonizes distinct sites within the urinary tract.

Both pathogenic and nonpathogenic bacteria encode complex systems for acquisition of environmental iron, an essential metal that is insoluble under neutral, aerobic conditions and protein bound within mammalian hosts. Uptake of iron in bacteria is achieved primarily through the synthesis, export, and uptake of small iron-chelating molecules termed siderophores. In Gram-negative bacteria, uptake of ferri-siderophores and heme iron is facilitated by specific outer membrane receptors. Ligand transport by these structurally conserved beta-barrel proteins is an energy-dependent process, requiring transduction of the proton motive force via the inner membrane complex TonB-ExbBD.

An essential role for iron acquisition by *Escherichia coli*, the causative agent of most community-acquired urinary tract infections (UTIs), has been well established. The genome of pyelonephritis *E. coli* strain CFT073 encodes 14 characterized outer membrane iron compound receptors, three siderophore biosynthesis systems, and a number of putative TonB-dependent receptors that may also be involved in iron acquisition (51). The genes encoding these iron acquisition systems were among the most highly upregulated genes in strain CFT073 during experimental infection of the murine urinary tract (46). Similarly, most of these systems, including the yersiniabactin and heme uptake loci, were highly expressed by clinical strains of *E. coli* in urine collected directly from women with cystitis

(14), implying a role for iron uptake during human UTI. Indeed, obtaining iron is a critical process for uropathogenic *E. coli* (UPEC) survival *in vivo*, as a *tonB* mutant was severely attenuated in a mouse pyelonephritis model, indicating that TonB-dependent receptors are required for UTI (49). Interestingly, asymptomatic bacteriuria strains, while lacking or containing mutations in classical UPEC virulence factors such as fimbriae, express the full complement of iron acquisition systems, providing further evidence for the necessity of iron uptake during successful urinary tract colonization (40).

UPEC strains produce up to four different siderophores: catecholates enterobactin and salmochelin, the hydroxamate aerobactin, and yersiniabactin, a mixed-type siderophore (20). Salmochelin, a glucosylated form of enterobactin that is resistant to binding and inactivation by the mammalian protein lipocalin-2 (11), also represents an immune evasion strategy. While enterobactin is produced and utilized by nearly all *E. coli* strains, genes encoding aerobactin, salmochelin, and yersiniabactin receptors are found more frequently among pathogens (5, 23, 25). Furthermore, increased yersiniabactin and salmochelin synthesis was detected in urinary *E. coli* isolates, compared to fecal isolates, of patients with recurrent UTI (20).

Although iron acquisition in general is required for UTI, no single uptake system that is necessary for this function has been identified. Although able to colonize the urinary tract during independent challenge, mutants defective for ChuA- or Hma-mediated heme uptake (15, 49), IroN-mediated salmochelin uptake (43), IutA-mediated aerobactin uptake (49), or Iha-mediated enterobactin uptake (22) were outcompeted by a wild-type strain during competitive infection. Similarly, UPEC strain CFT073 maintained the ability to colonize the murine

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Michigan Medical School, 5641 Medical Sciences Bldg. II, 1150 W. Medical Center Drive, Ann Arbor, MI 48109. Phone: (734) 764-1466. Fax: (734) 763-7163. E-mail: hmobley@umich.edu.

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Description ^a	Reference(s) or source
Strains		
CFT073	<i>E. coli</i> pyelonephritis isolate (Ent ⁺ Sal ⁺ Aer ⁺)	31
536	<i>E. coli</i> UTI isolate (Ent ⁺ Sal ⁺ Ybt ⁺)	2
Δ <i>fepA</i> mutant	CFT073 Δ <i>fepA</i> :: <i>cat</i> Cam ^r	This study
Δ <i>iha</i> mutant	CFT073 Δ <i>iha</i> :: <i>scaR</i> Kan ^s ; <i>kan</i> cassette removed	This study
Δ <i>iha</i> Δ <i>fepA</i> mutant	CFT073 Δ <i>iha</i> :: <i>scaR</i> Δ <i>fepA</i> :: <i>kan</i> Kan ^r ; <i>kan</i> cassette removed	This study
Δ <i>iroN</i> mutant	CFT073 Δ <i>iroN</i> :: <i>kan</i> Kan ^r or Δ <i>iroN</i> :: <i>scaR</i> Kan ^s ; <i>kan</i> cassette removed	This study
Δ <i>cirA</i> mutant	CFT073 Δ <i>cirA</i> :: <i>kan</i> Kan ^r	This study
Δ <i>fiu</i> mutant	CFT073 Δ <i>fiu</i> :: <i>cat</i> Cam ^r	This study
Δ <i>iutA</i> mutant	CFT073 Δ <i>iutA</i> :: <i>cat</i> Cam ^r or Δ <i>iutA</i> :: <i>scaR</i> Kan ^s ; <i>kan</i> cassette removed	This study
Δ <i>fhuA</i> mutant	CFT073 Δ <i>fhuA</i> :: <i>kan</i> Kan ^r	This study
Δ <i>fhuE</i> mutant	CFT073 Δ <i>fhuE</i> :: <i>cam</i> Cam ^r	This study
<i>chuA</i> mutant	CFT073 <i>chuA</i> :: <i>cat</i> Cam ^r	49
Δ <i>hma</i> <i>chuA</i> mutant	CFT073 Δ <i>hma</i> :: <i>kan</i> <i>chuA</i> :: <i>cat</i> Kan ^r Cam ^r	15
Δ <i>fepA</i> ₅₃₆ mutant	536 Δ <i>fepA</i> :: <i>cat</i> Cam ^r	This study
Δ <i>iroN</i> ₅₃₆ mutant	536 Δ <i>iroN</i> :: <i>scaR</i> Cam ^s ; <i>cat</i> cassette removed	This study
Δ <i>fhuA</i> ₅₃₆ mutant	536 Δ <i>fhuA</i> :: <i>scaR</i> Cam ^s ; <i>cat</i> cassette removed	This study
Δ <i>fhuE</i> ₅₃₆ mutant	536 Δ <i>fhuE</i> :: <i>cat</i> Cam ^r	This study
Δ <i>fyuA</i> ₅₃₆ mutant	536 Δ <i>fyuA</i> :: <i>kan</i> Kan ^r	This study
Δ <i>hma</i> Δ <i>chuA</i> ₅₃₆ mutant	536 Δ <i>hma</i> :: <i>kan</i> Δ <i>chuA</i> :: <i>cat</i> Kan ^r Cam ^r	This study
Δ <i>entF</i> ₅₃₆ mutant	536 Δ <i>entF</i> :: <i>kan</i> Kan ^r	This study
Δ <i>ybtS</i> ₅₃₆ mutant	536 Δ <i>ybtS</i> :: <i>kan</i> Kan ^r	This study
Plasmids		
pKD4	λ Red template vector; Kan ^r Amp ^r	7
pKD3	λ Red template vector; Cam ^r Amp ^r	7
pKD46	Red recombinase helper plasmid, temp sensitive; Amp ^r	7
pCP20	Flp recombinase helper plasmid, temp sensitive; Amp ^r	7
pGEN	pGEN-MCS, promoterless expression vector, p15A ori (copy no. ~15); <i>par hok sok mok parM parR</i> Amp ^r	13, 27
p _{native} <i>fyuA</i>	<i>fyuA</i> with native promoter (290 bp upstream) in pGEN; Amp ^r	This study
p _{native} <i>chuA</i>	<i>chuA</i> with native promoter (360 bp upstream) in pGEN; Amp ^r	This study
p _{native} <i>hma</i>	<i>hma</i> with native promoter (900 bp upstream) in pGEN; Amp ^r	15

^a Ent, enterobactin; Sal, salmochelin; Aer, aerobactin; Ybt, yersiniabactin; Kan, kanamycin; Cam, chloramphenicol; Amp, ampicillin.

urinary tract when either enterobactin or aerobactin synthesis was disrupted, but an *iucB entD* double mutant, defective for synthesis of both siderophores, was attenuated (49). Thus, functional redundancy exists among these iron acquisition systems.

Although both heme and siderophore iron acquisition contribute to the ability of UPEC to infect the urinary tract, evidence for distinct roles of specific systems exists. Heme uptake via ChuA was shown to play a role in bladder and kidney colonization (49), as well as in the formation of intracellular bacterial communities within superficial bladder epithelial cells (39), contributing to virulence more significantly than the heme receptor Hma (15). Mutants lacking the salmochelin receptor IroN, which were defective for urothelial cell invasion (10), were outcompeted by the wild-type strain in the bladders of infected mice, but kidney colonization was unaltered (43). Conversely, a mutant deficient in both enterobactin and aerobactin synthesis was only modestly attenuated in the bladder but showed a 1-log reduction in kidney colonization (49). Consequently, different iron acquisition systems appear to have differing contributions to bladder and kidney infection. Moreover, there is evidence for the unequal contributions of distinct iron acquisition systems to the virulence of other bacterial pathogens (29, 47, 50).

While there is substantial evidence for functional redundancy among UPEC iron acquisition systems, we hypothesized

that specific systems may have a greater impact on urinary tract colonization. In this study, we used a series of mixed infections, competing mutants defective for specific iron receptors against one another to directly assess the relative contributions of heme and siderophore receptors to UTI. The data presented here support a model of UPEC pathogenesis in which uptake of heme and uptake of noncatecholate siderophores play dominant roles at specific sites within the urinary tract.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains used in this study are listed in Table 1. Bacteria were routinely cultured in Luria broth (10 g/liter tryptone, 5 g/liter yeast extract, 0.5 g/liter NaCl) with appropriate antibiotics (100 μ g/ml ampicillin, 25 μ g/ml kanamycin, 20 μ g/ml chloramphenicol) at 37°C with aeration.

Mutant construction and cloning. Isogenic mutants were generated in *E. coli* CFT073 and 536 (Table 1) using the λ Red recombinase system (7). Briefly, kanamycin or chloramphenicol resistance cassettes were PCR amplified from the template plasmid pKD4 or pKD3, respectively, using primers containing regions identical to the 5' and 3' ends of the gene to be deleted (Table 2). The resulting product was used to replace >80% of the target gene by homologous recombination in bacteria expressing Red recombinase from pKD46. Flp recombinase expression from pCP20 was used to remove antibiotic resistance markers from the strains. All mutants were confirmed by PCR.

For complementation, the *fyuA* and *chuA* open reading frames (ORFs) plus ~300 bp upstream sequence were PCR amplified from *E. coli* 536 genomic DNA using EasyA polymerase (Agilent). Fragments containing *fyuA* and *chuA* were cloned into the SbfI-NcoI and EcoRI-EagI sites of pGEN (Table 1), respectively. The resulting constructs were verified by DNA sequencing.

TABLE 2. Primers for mutant construction and complementation^a

Gene or plasmid	Forward primer	Reverse primer
<i>fepA</i>	<u>GCCCGCGATGTATCGGAGATCATTCGTACCATGCCT</u> <u>GGCGGTGTAGGCTGGAGCTGCTTC</u>	<u>ACGTCCCGGCTCGTTATAGGTATACGCACCCGCA</u> <u>CCGGCGATGGGAATTAGCCATGGTCC</u>
<i>iha</i>	<u>CCACTCTGGCTCCGTAGTCATTCCTGTCTCGGATT</u> <u>TTCTGTGTAGGCTGGAGCTGCTTC</u>	<u>GTTGATGATCCCGTCTGGAAGTAATACCCGCAT</u> <u>ACAGCGATGGGAATTAGCCATGGTCC</u>
<i>iroN</i>	<u>CTCCGACGATGATAATGACGAGACTCTGGTGGTGG</u> <u>AAGCCGTGTAGGCTGGAGCTGCTTC</u>	<u>CGGCCTGGCTCGTTATAGGTATTCGCCCCCTTCAG</u> <u>AAGATCATGGGAATTAGCCATGGTCC</u>
<i>cirA</i>	<u>CCGTACGCAAGGGGACGTAAAGAAGATGTGAGCG</u> <u>ATACCCGTGTAGGCTGGAGCTGCTTC</u>	<u>CGTTATAGCTGTAGTCGTACGACTGAGATCCTT</u> <u>GTCGCCATGGGAATTAGCCATGGTCC</u>
<i>fiu</i>	<u>GCCGGTCTTTGTATTGGTATTACGCCTGTGGCACAG</u> <u>GCACGTGTAGGCTGGAGCTGCTTC</u>	<u>CTGTGAGCAAGAAGGTTCTTGGCTCGCCCCGGGT</u> <u>GATAACGATGGGAATTAGCCATGGTCC</u>
<i>iutA</i>	<u>CCCGGCTCTTGGTCCGCTGCTTCTTGTCTGTCTGT</u> <u>CACCGTGTAGGCTGGAGCTGCTTC</u>	<u>CCGGCCTTTGTAGTCGTACAGTGAAGCAGGGCC</u> <u>GTAACCCGATGGGAATTAGCCATGGTCC</u>
<i>fhuA</i>	<u>GTTGTAGTAGCCACAGCGTTAGCGGCATGTCTGT</u> <u>TTATGGTGTAGGCTGGAGCTGCTTC</u>	<u>CTGACGTTCTGCGCCCCAGAAGCAACCATAAGTG</u> <u>TTAAAGATGGGAATTAGCCATGGTCC</u>
<i>fhuE</i>	<u>ACATCAAGCTATCATCAAACCGCTCACTCCTTGCCGG</u> <u>TTGCGTGTAGGCTGGAGCTGCTTC</u>	<u>CGGTAATGCTGAAATTACGCGGTGCGCCGTAGA</u> <u>CGATAGATGGGAATTAGCCATGGTCC</u>
<i>chuA</i>	<u>CCGCAATTTACCTCGTTGCGTTTGTGTTGTTGGCT</u> <u>TTGGGTGTAGGCTGGAGCTGCTTC</u>	<u>CGTTACGACCATCTGTGGGATGCCTTGC GGCGA</u> <u>CCAGTAATGGGAATTAGCCATGGTCC</u>
<i>hma</i>	<u>GTCGAATTTATATCCTTTCCACTCTGTCAATTATGCAT</u> <u>TTCTGTGTAGGCTGGAGCTGCTTC</u>	<u>CAGAGATTATGTTGGCCTGACCCATCACAAGAGT</u> <u>ATGTTTATGGGAATTAGCCATGGTCC</u>
<i>fyuA</i>	<u>GGCTTTATCCTCTGGCCTTGGGGGGATTATTGCTCC</u> <u>CCGCCGTGTAGGCTGGAGCTGCTTC</u>	<u>CGTATTGATACCGACGGTGCAGCCCATATTGACC</u> <u>TGCGCGATGGGAATTAGCCATGGTCC</u>
<i>entF</i>	<u>GCCAGCATTTACCTTTGGTTCGCCGACAGCCCGGCA</u> <u>TCTGGTGTAGGCTGGAGCTGCTTC</u>	<u>CGCCACCGGAGAGATAATATCCACATGCGCACA</u> <u>ATCCTGACATGGGAATTAGCCATGGTCC</u>
<i>ybtS</i>	<u>GAATTTCTACATCTGGCGTTACCAGAGGAACAATG</u> <u>GCTACGTGTAGGCTGGAGCTGCTTC</u>	<u>ACCATTAATAGGGCGCAATGCTCGCTAATTTCT</u> <u>CCCGGGATGGGAATTAGCCATGGTCC</u>
<i>p_{native}fyuA</i>	<u>CCTGCAGGTGCCAACTGACTATC</u>	<u>CCATGGTGTAAAAGGGATACCTTTTCGGT</u>
<i>p_{native}chuA</i>	<u>CCGTACGAATCTTATTGTGTTAATGGCGG</u>	<u>GCTTTCCGGCCGTTACCATTGATAACTCACG</u>

^a Primer sequences are listed 5' to 3'. Underlined sequences in mutant construction primers are identical to regions at the 5' and 3' ends of the gene to be disrupted.

In vitro growth. CFT073 and 536 wild-type strains and isogenic mutants were cultured overnight in LB in the absence of antibiotics. Cultures were washed once in phosphate-buffered saline (PBS) and standardized to an optical density at 600 nm (OD₆₀₀) of ~1.0, and approximately 10⁵ CFU was inoculated into LB or LB containing 200 μM 2,2'-dipyridyl (DPD). Growth curves were determined using a Bioscreen C growth curve analyzer (Growth Curves) at 37°C with aeration.

Murine cochallenge model of ascending UTI. Six- to 8-week-old female CBA/J mice were inoculated transurethrally as previously described (21), with modification. Bacteria were cultured overnight in LB without antibiotics prior to inoculation. Each strain was harvested by centrifugation (3,500 × g, 30 min, 4°C) and resuspended in PBS to an OD₆₀₀ of about 4.0 (~10⁹ CFU/ml). Three to four strains were mixed in equal ratios, and 50 μl of this suspension (10⁸ CFU) was delivered to each mouse via a sterile 0.28-mm polyethylene catheter attached to an infusion pump (Harvard Apparatus). To quantify each strain in the inoculum, dilutions were plated onto LB agar with and without appropriate antibiotics to differentiate antibiotic-sensitive and -resistant mutants. Seventy-two hours post-inoculation (hpi), organs were removed from euthanized mice and homogenized in 3 ml PBS using a GLH homogenizer (Omni International). Using an Auto-plate 4000 spiral plater (Spiral Biotech), tissue homogenate was plated onto LB agar containing appropriate antibiotics. Colonies were enumerated with a QCount automated plate counter (Spiral Biotech) and CFU/g tissue determined (output). For cochallenge experiment (two inoculated strains), competitive indices were calculated by dividing the ratio of *ΔfepA* to *Δiha* mutant in the output by the ratio of the two mutants in the inoculum [(CFU_{*fepA*}/CFU_{*iha*})_{output}/(CFU_{*fepA*}/CFU_{*iha*})_{input}]. For multistrain coinfections, fitness indices (FI) for each organ sample were calculated by dividing the fraction of each mutant in the output by the fraction of each mutant in the inoculum [(CFU_{mutant}/CFU_{total})_{output}/(CFU_{mutant}/CFU_{total})_{input}].

RNA isolation and qPCR. For *in vitro* measurements, strains were cultured with aeration to late exponential phase (OD₆₀₀ of 0.5 to 0.6) in 100 ml LB with or without 200 μM DPD and 10 μM hemin. Culture samples (200 μl) were mixed with 25 μl cold 5% phenol-ethanol stop solution, pelleted (1 min, 10,000 × g, 4°C), and stored at -80°C for RNA isolation. For *in vivo* samples, CBA/J mice (*n* = 15) were infected with *E. coli* strain CFT073 or 536 as described above. From 6 to 72 hpi, urine was collected periodically and pooled from each cage of

mice (5 animals). Immediately after collection, cold 5% phenol-ethanol stop solution was added to each pool (0.125 μl solution per μl urine), and samples were pelleted (1 min, 10,000 × g, 4°C) and stored at -80°C. For RNA isolation, bacterial pellets (collected from five mice) were pooled from all time points. Thawed pellets were resuspended in 100 μl RNase-free Tris-EDTA (TE) containing 1 mg/ml lysozyme and RNA isolated using the RNeasy protocol (Qiagen). Samples were DNase treated according to the Turbo DNA-Free procedure (Ambion) and cDNA synthesized using SuperScript II first-strand synthesis reagents (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed using 30 ng cDNA template and Brilliant SYBR green reagents (Agilent). Data were normalized to the *gapA* transcript and analyzed using MxPro 4.0 software (Agilent).

In vitro competition assay. Cocultures were performed as previously described (28). Overnight cultures of CFT073 *ΔfhuA*, *ΔfhuE*, and *ΔiutA* mutants were diluted 1:100 in LB and cultured to late exponential phase. Culture samples (1 ml) were pelleted (1 min, 10,000 × g, room temperature), resuspended in 1 ml PBS, and standardized to an OD₆₀₀ of 1.0. Cell suspensions were mixed 1:1:1, diluted 1:100 into 5 ml LB with or without 200 μM DPD, and incubated at 37°C. Every 8 or 16 h, cultures were passaged 1:100 or 1:500 into 5 ml fresh medium, respectively. At 24, 48, and 72 h, cultures were plated on LB agar and LB containing kanamycin or chloramphenicol to enumerate each mutant.

Iron source utilization assays. The ability of complemented mutants to utilize various iron sources was determined as previously described (48), with modifications. Supernatants from the *ΔentF*₅₃₆ or *ΔybtS*₅₃₆ mutant, cultured overnight with 100 μM DPD, were filter sterilized and concentrated 10-fold using a vacuum centrifuge. Overnight cultures of wild-type 536 and *ΔfhuA*₅₃₆ and *Δhma* *ΔchuA*₅₃₆ mutants carrying pGEN constructs were diluted 1:100 into LB containing 200 μM DPD and cultured for ~6 h at 37°C. One-milliliter samples of iron-limited cultures were pelleted (1 min, 10,000 × g, room temperature), resuspended in 1 ml PBS, and adjusted to an OD₆₀₀ of 1.0. Bacterial suspension was added to molten sorbitol-MacConkey agar containing 350 or 375 μM DPD to ~5 × 10⁴ CFU/ml. Using the ends of pipette tips, ~8-mm diameter wells were punched into solidified plates, and 80 μl of 100 μM FeCl₂, 100 μM hemin, or concentrated supernatant was added to each well (6). Plates were imaged, and diameter of growth surrounding each well was measured after 72 h at 37°C.

TABLE 3. UPEC iron receptors and summary of receptor mutant fitness during competitive mixed infection

Gene	Locus tag	Receptor substrate	Substrate type	Reference	Gene presence		No. of FI <1 ^a	
					CFT073	536	Bladder	Kidney
<i>fepA</i>	c0669	Enterobactin	Catecholate	54	+	+	0/5	1/5
<i>iha</i>	c3610	Enterobactin	Catecholate	30	+	—	0/5	0/5
<i>iroN</i>	c1250	Salmonochelin	Catecholate	19	+	+	1/4	0/4
<i>fhu</i>	c1374	DBS, DHB ^b	Catecholate	17	+	+	0/1	0/1
<i>cirA</i>	c2690	DBS, DHB	Catecholate	17	+	+	0/1	0/1
<i>iutA</i>	c3623	Aerobactin	Hydroxamate	8	+	—	2/3	3/3
<i>fhuA</i>	c0185	Ferrichrome	Hydroxamate	24	+	+	0/2	0/2
<i>fhuE</i>	c1374	Coprogen, rhodotorulic acid	Hydroxamate	18	+	+	0/2	0/2
<i>fyuA</i>	c2436	Yersiniabactin	Mixed	38	+	+	2/2	1/2
<i>chuA</i>	c4308	Heme	NA ^d	48	+	+	1/3	3/3
<i>hma</i>	c2482	Heme	NA	15	+	+	1/3	3/3

^a Number of times a mutant defective for the indicated gene was outcompeted (fitness index significantly less than 1.0) per number of mixed infections in which it was included (both CFT073 and 536 backgrounds). Values for *fepA* and *iha* both include results for the $\Delta iha \Delta fepA$ double mutant, and values for *chuA* and *hma* reflect data from the $\Delta hma \Delta chuA$ double mutant.

^b DBS, dihydroxybenzoylserine; DHB, dihydroxybenzoate.

^c CFT073 carries an intact *fyuA* gene, but nonsense mutations and a 711-bp insertion element in genes encoding yersiniabactin biosynthesis machinery (*irp1* and *irp2*) prevent siderophore production.

^d NA, not applicable.

Statistical analyses. Statistical analyses were performed using GraphPad Prism 5. For competitive infections, significance was determined using the Wilcoxon rank sum test on \log_{10} -transformed fitness indices or competitive indices with a hypothetical median of 0 ($\log_{10}1 = 0$). The Mann-Whitney test was used to compare fitness indices for the same mutant between different organs. Correlations were determined using the Spearman rank test.

RESULTS

Heterologous iron receptor gene expression is unaltered in specific receptor mutant strains. To examine the contributions of heme and siderophore uptake to UTI, deletion mutations in individual iron receptor genes were made in UPEC strain CFT073. To simplify analysis, only receptors having defined substrates were included (Table 3). Growth of all mutants was examined during culture in LB and in the presence of 200 μ M DPD. All mutants had growth rates similar to that of the isogenic parent strain both in rich medium and under iron limitation (data not shown). Previous work from our laboratory has shown that expression of iron receptor genes in CFT073 is upregulated under iron-limiting conditions, both *in vitro* (1) and *in vivo* (46). As expression of these genes is likely Fur regulated, they are predicted to be expressed independently of one another. Nonetheless, to determine whether receptor gene expression was dysregulated in the receptor mutants, transcripts isolated from selected mutants, cultured under iron limitation, were measured by qPCR. With the exception of the deleted genes themselves, none of the receptor genes examined differed by more than 2-fold from wild-type expression levels (Fig. 1). Lack of *chuA* “downregulation” by the *chuA* and $\Delta hma \Delta chuA$ mutants is due to the fact that, rather than gene deletion, these constructs contain an insertion mutation in *chuA* (Table 1) and retain the entire *chuA* coding sequence. Although expression of *iroN* appeared slightly elevated in the $\Delta iutA$ mutant, expression of *btuB*, the vitamin B12 receptor included as a control, was also slightly elevated, suggesting that this difference is not biologically significant. For heme receptor Δhma (data not shown), *chuA*, and $\Delta hma \Delta chuA$ mutants, heterologous receptor expression was unaltered in both the absence (Fig. 1) and the presence (data not shown) of

excess hemin. Thus, deletion of one or more outer membrane iron receptor genes does not affect transcript abundance of the remaining receptor genes, indicating that any defects in mutant fitness are not compensated for by upregulation of heterologous receptor genes.

Catecholate siderophore receptor mutants are equally fit in the murine urinary tract. Previous work from our laboratory demonstrated that a *chuA* heme receptor mutant was outcompeted by an *hma* heme receptor mutant in a murine model of UTI (15), indicating that receptors with similar substrate specificities can have differing contributions to UPEC colonization. Because *E. coli* CFT073 encodes multiple receptors that import the catecholate siderophore enterobactin, we hypothesized that they may contribute unequally to UTI. To test this, mice were challenged transurethrally with a 1:1 mixture of a $\Delta fepA$ mutant and a mutant lacking the IrgA homolog adhesin gene *iha*, which is carried outside the *ent fep* locus and transports both enterobactin and its iron-chelating linear degrada-

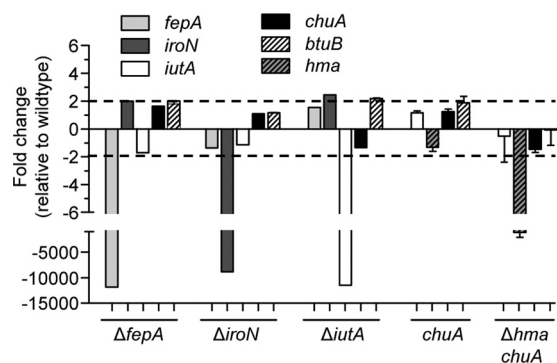


FIG. 1. Heme and siderophore receptor expression levels by qPCR in receptor mutants. RNA was isolated from wild-type CFT073 and receptor mutants (indicated on x axis) cultured in LB containing 200 μ M DPD. Transcript levels were measured in cDNA preparations from each strain and normalized to the *gapA* level, and results are shown as fold change relative to wild-type levels. Dashed lines represent 2-fold change and bars show the means.

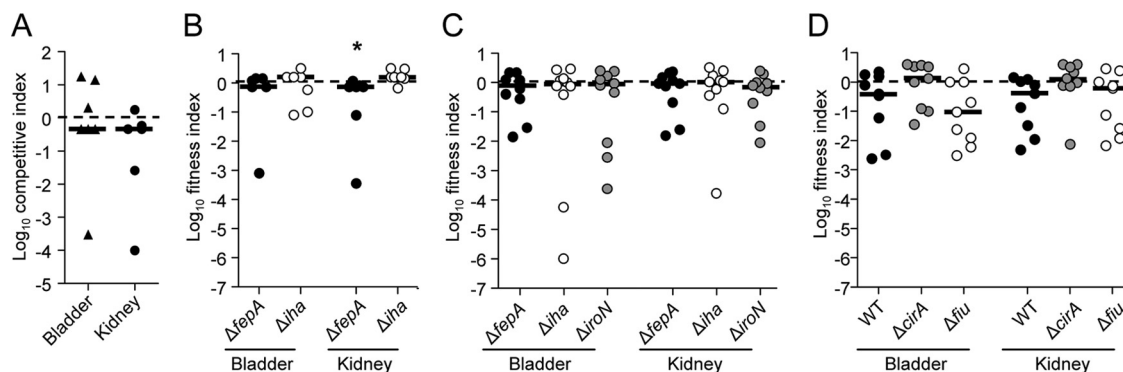


FIG. 2. Fitness of *E. coli* CFT073 catechol siderophore receptor mutants during mixed infections. (A) Competitive indices from cochallenge in CBA/J mice transurethrally challenged with a mixture of equal amounts of $\Delta fepA$ and Δiha mutants ($n = 7$). The total inoculum equaled 1×10^8 CFU/mouse. (B to D) Fitness indices calculated from the cochallenge results shown in panel A (B) or from mixed infections in mice inoculated with a mixture of $\Delta fepA$, Δiha , and $\Delta iroN$ mutants ($n = 10$) (C) or wild-type CFT073 (WT) and $\Delta cirA$ and Δfiu mutants ($n = 9$) (D). Fitness indices at 72 hpi were calculated by dividing the fraction of each mutant in the output (CFU/g tissue) by the fraction of each mutant in the input (CFU/ml inoculum). Bars represent the medians, and the dashed line indicates the theoretical fitness index of 1.0 ($\log_{10} 1 = 0$). *, $P = 0.0333$.

tion product dihydroxybenzoylserine (DBS) (30). Competitive indices, calculated at 72 hpi, indicated that both mutants were equally fit in the murine urinary tract (Fig. 2A), although the $\Delta fepA$ mutant tended to be outcompeted by the Δiha mutant in the kidney ($P = 0.0502$).

Salmochelin, imported by the receptor IroN, is a glucosylated form of enterobactin shown to resist sequestration by the host protein lipocalin-2 (11) and contribute to UPEC pathogenesis (43). In addition to salmochelin, IroN was demonstrated to transport enterobactin and DBS (37). Therefore, we speculated that salmochelin uptake via the receptor IroN may contribute more significantly to UPEC pathogenesis than FepA or Iha, the substrates of which are susceptible to lipocalin-2 inactivation. Overall, we reasoned that, while mutants defective for each of these receptors displayed fitness defects when competed against a wild-type parent strain (22, 43), the receptors may have unequal contributions to *in vivo* fitness that would not be apparent during traditional cochallenge experiments. To examine this possibility directly, a three-way *in vivo* competition assay was performed with CFT073 $\Delta fepA$, Δiha , and $\Delta iroN$ mutants. Mutants were mixed at a 1:1:1 ratio and coinoculated transurethrally into CBA/J mice. However, because more than two strains were inoculated, we reasoned that calculation of traditional competitive indices would not take into account the competition occurring among all three mutant strains. Therefore, we developed a modified competitive index, which we termed the fitness index (FI), that compared the fraction of each mutant recovered from each organ to the fraction of the same mutant in the inoculum [(CFU_{mutant}/CFU_{total})_{output}/(CFU_{mutant}/CFU_{total})_{input}]. When the cochallenge data presented in Fig. 2A were reanalyzed by this new measure, similar results were obtained (Fig. 2B). However, FI values for the $\Delta fepA$, Δiha , and $\Delta iroN$ mutants were not significantly different from 1.0 ($\log_{10} 1.0 = 0$) (Fig. 2C), indicating that all three mutants were equally fit in the murine urinary tract when placed in direct competition.

Two additional outer membrane receptors, CirA and Fiu, have been shown to transport the enterobactin breakdown products DBS and dihydroxybenzoate (DHB) (17). While roles for *iha*, *iroN*, and *fepA* (*ent*) in UPEC (22, 43, 49) have been

demonstrated, the potential contributions of *cirA* and *fiu* are unknown. Before their relative contributions to UPEC colonization could be assessed, we first sought to test whether they played any role in UTI. Thus, isogenic $\Delta cirA$ and Δfiu mutants were coinoculated with wild-type CFT073. Surprisingly, neither mutant displayed a significant decrease in fitness when competed against the wild type (Fig. 2D), indicating that neither *cirA* nor *fiu* contributes significantly to UTI in this model. Consequently, they were not tested for their ability to compete against the other catechol siderophore receptors. Together, these data demonstrate that the catechol siderophore receptors FepA, Iha, and IroN contribute equally to UPEC colonization of the murine urinary tract.

Aerobactin receptor mutant is outcompeted *in vivo* but not *in vitro*. While *E. coli* encodes several outer membrane receptors for the import of catechol siderophores, a single receptor, IutA, is responsible for uptake of the hydroxamate siderophore aerobactin. Most *E. coli* strains, including CFT073, can also utilize the fungal hydroxamate siderophores ferrichrome, coprogen, and rhodotorulic acid, via the receptors FhuA and FhuE (18). Although we predicted that these xenosiderophore receptors would not be functional in the urinary tract in the absence of fungal species, we used the *in vivo* competition assay to measure the relative contributions of hydroxamate siderophore receptors to infection. As expected, the $\Delta iutA$ mutant was significantly outcompeted in both the bladder ($P = 0.0020$) and the kidneys ($P = 0.0371$) (Fig. 3A), indicating that IutA is the most significant hydroxamate receptor during UTI. Interestingly, although the $\Delta iutA$ mutant was outcompeted in both organs, its bladder fitness indices were significantly lower than those in the kidney ($P = 0.0232$), suggesting that this receptor contributes most during bladder colonization and that nonaerobactin iron sources compensate for the loss of *iutA* during kidney infection. In contrast to *in vivo* competition, all hydroxamate receptor mutants were maintained at nearly equivalent ratios when cocultured in an *in vitro* competition assay (28), both during growth under iron-limited conditions (Fig. 3B) and in rich medium (data not shown). This suggests that the multimutant competition infection approach

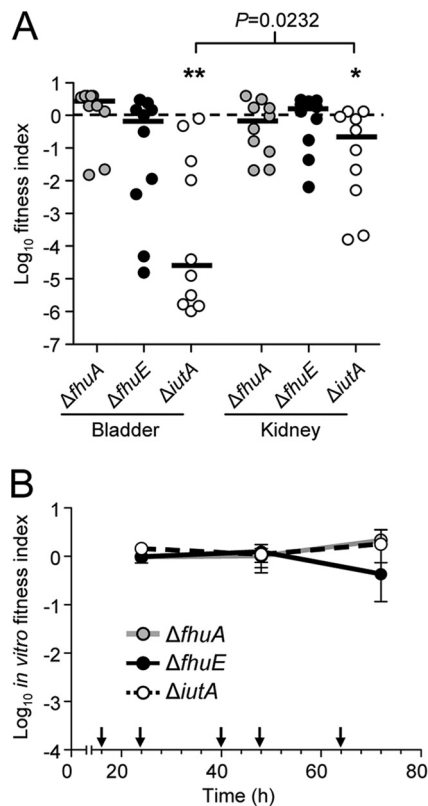


FIG. 3. *In vivo* and *in vitro* competitive fitness of *E. coli* CFT073 hydroxamate receptor mutants. (A) *In vivo* competition in CBA/J mice transurethrally challenged with a mixture of equal amounts of $\Delta fhuA$, $\Delta fhuE$, and $\Delta iutA$ mutants ($n = 10$). Fitness indices at 72 hpi were calculated by dividing the fraction of each mutant in the output (CFU/g tissue) by the fraction of each mutant in the input (CFU/ml inoculum). Bars represent the medians, and the dashed line indicates the theoretical fitness index of 1.0. The P value displayed indicates significance by the Mann-Whitney test. *, $P = 0.0371$, and **, $P = 0.0020$, by the Wilcoxon signed-rank test. (B) *In vitro* culture competition of $\Delta fhuA$, $\Delta fhuE$, and $\Delta iutA$ mutants cultured in LB containing 200 μ M DPD. After inoculation, cultures were passaged into fresh medium every 8 (1:100 dilution) or 16 (1:500 dilution) h, indicated by arrows. Means of triplicate cultures are plotted and error bars show standard errors of the means. Fitness indices, calculated as described for panel A, are shown.

is capable of specifically identifying mutants with *in vivo* fitness defects.

Aerobactin and heme acquisition contributes most significantly to UTI. To directly assess the relative contributions of aerobactin, salmochelin, and enterobactin siderophore uptake, $\Delta iroN$, $\Delta fepA$, and $\Delta iutA$ mutants were competed in a mixed infection of the murine UTI model. As observed in Fig. 2C, neither the $\Delta iroN$ mutant nor the $\Delta fepA$ mutant was significantly underrepresented in the total bacterial output of the bladder or kidneys, suggesting that they contribute relatively equally to virulence (Fig. 4A). In contrast, in both the bladder and the kidneys, the $\Delta iutA$ mutant was significantly outcompeted ($P = 0.0059$ and $P = 0.0098$, respectively), indicating that *iutA* contributes more to UPEC iron uptake during urinary tract colonization than either *iroN* or *fepA*.

In addition to receptors for ferrisiderophore uptake, *E. coli* CFT073 encodes two TonB-dependent heme receptors, ChuA

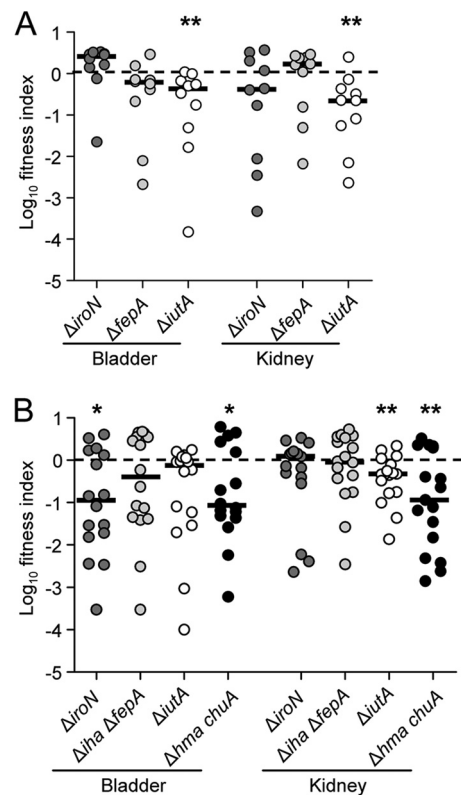


FIG. 4. Fitness of *E. coli* CFT073 siderophore and heme receptor mutants during mixed infection. *In vivo* competition in CBA/J mice transurethrally challenged with a mixture of $\Delta iutA$, $\Delta iroN$, and $\Delta fepA$ mutants ($n = 10$) (A) or $\Delta iroN$, $\Delta iha \Delta fepA$, $\Delta iutA$, and $\Delta hma chuA$ mutants ($n = 17$) (B). Fitness indices at 72 hpi were calculated by dividing the fraction of each mutant in the output (CFU/g tissue) by the fraction of each mutant in the input (CFU/ml inoculum). Bars represent the medians, and the dashed line indicates the theoretical fitness index of 1.0. *, $P < 0.02$; **, $P < 0.01$.

and Hma, and requires at least one for full virulence (15). To establish the role for heme acquisition in the context of siderophore utilization, a heme uptake-deficient $\Delta hma chuA$ double mutant was included in a four-way competition infection with the aerobactin and catechololate receptor mutants. To control for potential redundancy effects that may decrease the fitness of a mutant lacking two outer membrane iron receptors, a $\Delta iha \Delta fepA$ double mutant lacking the two known enterobactin receptors was constructed and included in the mixed infection. In both the bladders and the kidneys of infected mice, the $\Delta hma chuA$ mutant was outcompeted by the coinoculated mutants ($P = 0.0121$ and $P = 0.0052$, respectively), while the $\Delta iha \Delta fepA$ mutant was not (Fig. 4B), demonstrating that Hma and ChuA contribute more to UPEC colonization than Iha and FepA. Similarly to our previous observations, the $\Delta iutA$ mutant was significantly underrepresented in the kidney after a 72-h infection ($P = 0.0060$), again suggesting the relative importance of aerobactin utilization. However, in contrast to the fitness of the $\Delta iroN$ mutant during cochallenge with the $\Delta iutA$ and $\Delta fepA$ mutants (Fig. 4A), here the $\Delta iroN$ mutant was outcompeted in the bladder ($P = 0.0140$), indicating that the magnitude of the competitive advantage provided by *iroN* depends on the relative fitness of the coinoculated mutants.

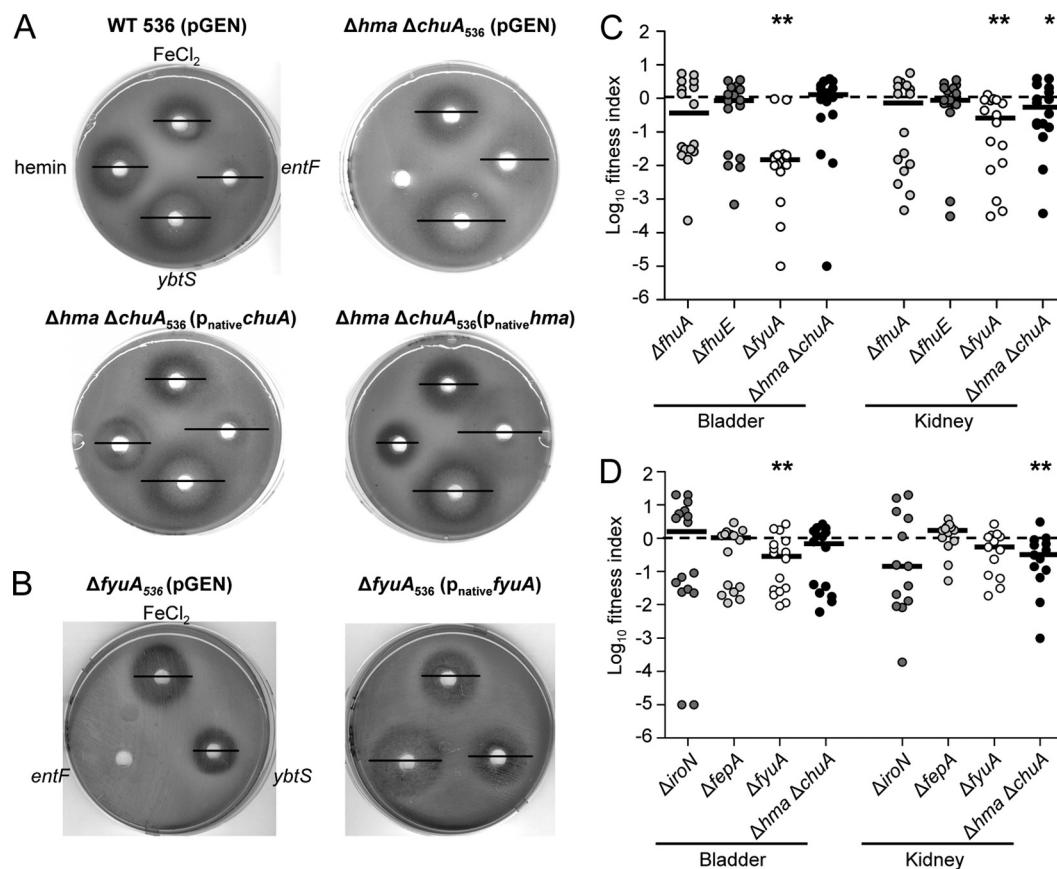


FIG. 5. Relative contribution of yersiniabactin uptake to *E. coli* UTI. (A) Growth of the *E. coli* 536 wild type carrying pGEN vector (top left) or the $\Delta hma \Delta chuA_{536}$ mutant carrying pGEN vector (top right), $p_{native} chuA$ (bottom left), or $p_{native} hma$ (bottom right) around wells containing (clockwise from top) 100 μ M $FeCl_2$, concentrated supernatant from the $\Delta entF_{536}$ mutant, concentrated supernatant from the $\Delta ybtS_{536}$ mutant, or 100 μ M hemin. Bars indicate zones of growth. (B) Growth of the *E. coli* $\Delta fyuA_{536}$ mutant carrying pGEN vector (left) or $p_{native} fyuA$ (right) around wells containing 100 μ M $FeCl_2$ or concentrated supernatants from the $\Delta ybtS_{536}$ or the $\Delta entF_{536}$ mutant. Bars indicate zones of growth. (C and D) *In vivo* competition in CBA/J mice transurethrally challenged with a mixture of *E. coli* 536 $\Delta fhuA_{536}$, $\Delta fhuE_{536}$, $\Delta fyuA_{536}$, and $\Delta hma \Delta chuA_{536}$ mutants ($n = 16$) (C) or $\Delta ironN_{536}$, $\Delta fepA_{536}$, $\Delta fyuA_{536}$, and $\Delta hma \Delta chuA_{536}$ mutants ($n = 13$) (D). Fitness indices at 72 hpi were calculated by dividing the fraction of each mutant in the output (CFU/g tissue) by the fraction of each mutant in the input (CFU/ml inoculum). Bars represent the medians, and the dashed line indicates the theoretical fitness index of 1.0. *, $P < 0.05$; **, $P < 0.01$.

These data taken together suggest that heme utilization via Hma and ChuA and aerobactin uptake via IutA contribute most significantly to UPEC iron acquisition during UTI.

Yersiniabactin and heme uptake is most critical for colonization by *E. coli* 536. While *E. coli* CFT073 produces three siderophores, enterobactin, salmochelin, and aerobactin, the majority of UPEC strains produce a fourth siderophore, yersiniabactin, often instead of aerobactin (20). Because CFT073 does not produce yersiniabactin, UPEC strain 536 was used to assess the relative contribution of this mixed-type siderophore to UTI. Isogenic receptor mutants were constructed in strain 536 and iron source utilization assays performed to confirm their ability to mediate heme- and siderophore-dependent growth. As expected, while wild-type 536 was capable of using $FeCl_2$, hemin, enterobactin/salmochelin (supernatants from a yersiniabactin-deficient $\Delta ybtS_{536}$ mutant), or yersiniabactin (supernatants from an enterobactin- and salmochelin-deficient $\Delta entF_{536}$ mutant) as a sole iron source (Fig. 5A), a $\Delta fyuA_{536}$ mutant could not grow utilizing supernatants from a $\Delta entF_{536}$ mutant (Fig.

5B). This defect in yersiniabactin utilization could be complemented by expression of *fyuA* in *trans*. Similarly to our findings with CFT073 (15), a $\Delta hma \Delta chuA_{536}$ double mutant was defective for hemin utilization and could be complemented with plasmids carrying either *hma* or *chuA* (Fig. 5A).

Results obtained from strain CFT073 suggest that aerobactin and heme utilization contributes most significantly to UTI (Fig. 4), and so the relative role of the yersiniabactin receptor FyuA was first examined in the context of heme and hydroxamate receptor function. Mice were challenged transurethrally with a mixture of equal amounts of $\Delta fhuA_{536}$, $\Delta fhuE_{536}$, $\Delta fyuA_{536}$, and $\Delta hma \Delta chuA_{536}$ mutants, and the fitness of each mutant was examined at 72 hpi. As expected, the $\Delta fhuA_{536}$ and $\Delta fhuE_{536}$ fungal hydroxamate receptor mutants had fitness indices that did not significantly differ from 1.0 (Fig. 5C), confirming the observations made with CFT073, suggesting that these receptors are relatively dispensable for UTI (Fig. 3A). In the bladder, only the $\Delta fyuA_{536}$ mutant was outcompeted ($P = 0.0020$), indicating that, of the four mutants tested, the yersinia-

bactin receptor represented the most contributive iron receptor to UPEC 536 colonization. However, both the $\Delta fyuA_{536}$ and $\Delta hma \Delta chuA_{536}$ mutants were outcompeted in the kidney ($P = 0.0098$ and $P = 0.0273$, respectively), suggesting that yersiniabactin and heme receptors contributed relatively equally to infection of this organ.

In addition to yersiniabactin, pyelonephritis strain 536 produces enterobactin and salmochelin. To examine the role of FyuA in the context of these siderophores, $\Delta fepA_{536}$, $\Delta iroN_{536}$, $\Delta fyuA_{536}$, and $\Delta hma \Delta chuA_{536}$ mutants were coinoculated, and their relative fitness levels were measured in a mixed-infection competition. Because *E. coli* 536 does not carry *iha* (4), the $\Delta fepA_{536}$ single mutant is equivalent to the CFT073 $\Delta iha \Delta fepA$ double mutant, at least with respect to known enterobactin receptors. As observed for CFT073, the FepA receptor appeared to play a lesser role in *E. coli* 536 colonization; the fitness index of the $\Delta fepA_{536}$ mutant was not different from 1.0 (Fig. 5D). The $\Delta iroN_{536}$ salmochelin receptor mutant was also able to compete well in both the bladder and the kidney. While the $\Delta fyuA_{536}$ mutant was outcompeted in the bladder ($P = 0.0057$) but not the kidney, the opposite pattern was observed for the $\Delta hma \Delta chuA_{536}$ mutant, as it was outcompeted only in the kidney ($P = 0.0046$). Together, these data demonstrate that FyuA-mediated yersiniabactin uptake and heme acquisition via Hma and ChuA are most critical for murine urinary tract colonization by UPEC strain 536.

Heme and noncatecholate siderophore receptors contribute most significantly to UTI. As expected, the relative competitive fitness of each iron receptor mutant depends, to some extent, on the other mutants included in the mixed infection. However, comparison of all fitness indices of both CFT073 and 536 mutants from the array of mixed-infection competitions conducted revealed several patterns. First, the salmochelin and enterobactin receptors appeared individually to contribute the least to urinary tract colonization, as mutants lacking these genes were rarely outcompeted (Table 3). In contrast, the $\Delta iutA$, $\Delta fyuA$, and $\Delta hma \Delta chuA$ mutants were the most frequently outcompeted. All had fitness indices less than 1.0 at least half of the times they were tested, regardless of coinoculated mutants, suggesting that these receptors contribute most significantly to UTI by either *E. coli* strain CFT073 or 536. Finally, tissue specificity was also observed, as a $\Delta hma \Delta chuA$ mutant displayed defective bladder fitness in only 1 of 3 inoculum mixtures, while it was always outcompeted during kidney infection (Table 3). Thus, it appears that heme and noncatecholate receptors contribute most significantly to UPEC iron acquisition during colonization of the murine urinary tract.

Relative receptor contribution to virulence partially correlates with *in vivo* expression. Previous work from our laboratory indicated that, for *hma* and *chuA*, receptor expression correlated with relative contribution to heme uptake and virulence (15). To determine whether differences in siderophore receptor gene expression may account for the observed differences in competitive fitness, receptor transcript levels were measured by qPCR from bacteria isolated from the urine of infected mice. All iron receptor genes examined were upregulated by CFT073 or 536 during murine UTI or culture in iron-limited medium, compared to culture in rich medium (data not shown). The relative transcript levels of

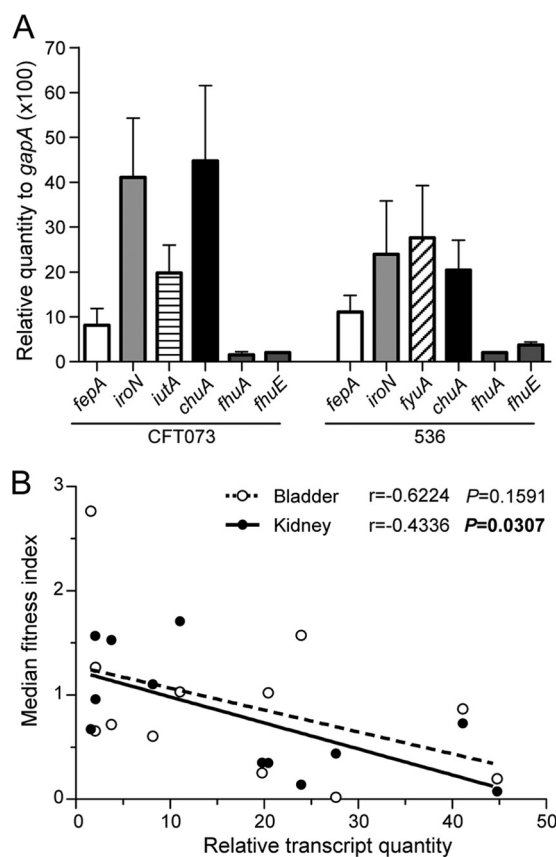


FIG. 6. Expression of iron receptor genes by UPEC during murine experimental UTI. (A) Relative quantities of UPEC iron receptor transcripts, normalized to that of the *gapA* transcript, present in the urine of mice inoculated transurethrally with 1×10^8 CFU of wild-type CFT073 or 536. Bars represent the means of duplicate or triplicate samples, with each sample pooled from five mice over 6 to 72 hpi, and error bars show the standard errors of the means. (B) Mean relative transcript quantity shown in panel A plotted against the median fitness index of the corresponding mutant during bladder or kidney infection by CFT073 or 536. The FI value for each gene/mutant is the median of all mixed infections shown. For the CFT073 $\Delta fepA$ mutant, the FI value is the median of the $\Delta fepA$ and $\Delta iha \Delta fepA$ mutant FIs combined. Best-fit lines, Spearman correlation coefficients (*r*), and corresponding *P* values are shown.

the receptors varied, though. In CFT073, transcripts for *chuA* and *iroN* were the most abundant, followed by that for *iutA*, while *iroN*, *fyuA*, and *chuA* transcripts had the highest levels in *E. coli* 536 (Fig. 6A). While expression of *chuA* and *fyuA* appears to correlate with the relatively high contribution of these genes to UTI, expression of *iroN* does not, as the *iroN* mutant was outcompeted in only 1 of 4 mixed-infection competitions (Table 1). When the median fitness index of each mutant was compared to the relative transcript abundance of the corresponding iron receptor gene, a significant correlation ($P = 0.0307$) was found in the kidneys but not the bladder (Fig. 6B). Iron receptor genes with higher expression levels tended to contribute more significantly to kidney infection than those with lower expression levels (Fig. 4, 5, and 6B). However, there were notable exceptions to this tendency, including *iroN*, suggesting that

additional factors also contribute to the relative importance of each iron receptor to UPEC pathogenesis.

DISCUSSION

Here we demonstrate nonequivalent roles for distinct iron acquisition systems during urinary tract colonization by UPEC and provide evidence for a functional hierarchy of these systems. Enterobactin and salmochelin receptors FepA, Iha, and IroN generally supplied redundant functions and contributed less to virulence and, presumably, iron uptake *in vivo* than noncatecholate siderophore and heme receptors. This hierarchy correlated with receptor gene expression for kidney, but not bladder, colonization. Overall, our data suggest a model for UPEC pathogenesis in which heme receptors Hma and ChuA and the noncatecholate siderophore receptor IutA or FyuA provide the most critical iron acquisition functions in the urinary tract.

In contrast to traditional coinfection studies, which compete a mutant against its wild-type parent strain, this study utilized a series of mixed infections that competed groups of mutants against one another, directly evaluating their relative roles in infection. We reasoned that mutants lacking iron receptors that contributed more significantly to uropathogenesis would be outcompeted by mutants lacking receptors whose functions were less important. To our knowledge, this is the first report to utilize direct competition between groups of mutants to examine the utility or redundancy of distinct iron acquisition systems in *E. coli* or other bacterial species; however, *in vivo* competition between two mutants has been used by our and other laboratories to examine the contribution of heme receptors (15, 49) and TonB systems (45) to UPEC and *Vibrio cholerae* pathogenesis, respectively. Related approaches using up to three coinoculated strains, often including double or triple mutants, have also been used in other infection models to assess the roles of *Proteus mirabilis* fimbriae (55) and *Streptococcus pneumoniae* oligopeptide transporters (26). We propose that this mixed mutant coinfection approach may be useful to dissect other redundant or partially redundant systems in UPEC or other bacterial pathogens.

While the approach used here is novel, several groups have investigated the relative importance of different iron acquisition systems to bacterial virulence. Ornibactin was concluded to be the most important siderophore for *Burkholderia cenocepacia*, as an ornibactin uptake mutant was cleared from a rat lung model, while a mutant lacking pyochelin uptake was maintained at wild-type levels (50). Similarly to our results, yersiniabactin was found to contribute more substantially than enterobactin to murine intranasal infection with *Klebsiella pneumoniae* (29). Miller and colleagues speculated that acquisition of nonenterobactin siderophore systems may be a critical component of enterobacterial pathogenesis, a hypothesis supported by our findings with UPEC.

The results presented here indicate that uptake of either aerobactin or yersiniabactin is more important to UPEC colonization of the urinary tract than enterobactin or salmochelin utilization. In strain CFT073, this disparity was so pronounced that the loss of the IutA aerobactin receptor was more detrimental to competitive fitness than the loss of two enterobactin receptors (FepA and Iha). While differences in receptor tran-

script abundance correlated with this hierarchy during kidney infection, this was not the case during bladder colonization, indicating that additional factors likely contribute to these observations. The difference cannot be explained simply by siderophore affinity, as the affinity of enterobactin for ferric iron (K_d [dissociation constant] = 10^{-52} M) is substantially higher than that of aerobactin (K_d = 10^{-23} M) or yersiniabactin (K_d = 10^{-36} M) (33, 35). Furthermore, while the low contribution of enterobactin to UPEC virulence may be explained by its susceptibility to sequestration by lipocalin-2 (12), salmochelin is resistant to this inactivation (11) and would therefore be expected to play a greater role in urinary tract colonization.

Previous biochemical studies examining the properties of aerobactin and enterobactin may provide insight into these results. Aerobactin was shown to be more effective than enterobactin at delivering iron to *E. coli* at low siderophore concentrations (53), as would be expected in a dynamic urinary tract environment. Aerobactin was also found to be excreted from *E. coli* more rapidly than enterobactin, and each siderophore's efficiency varied with the type and amount of iron chelator used in the culture medium (9). Moreover, aerobactin, which has long been associated with invasive *E. coli* (52), demonstrated a preference for chelating and delivering host cell-associated, rather than transferrin-bound, iron (3). Thus, it appears that the urinary tract environment and the intimate association of UPEC with the urothelium, perhaps during invasion and formation of intracellular bacterial communities (32), may favor aerobactin (and perhaps yersiniabactin) function over enterobactin activity. It remains to be tested whether these properties of enterobactin are also characteristic of salmochelin, which is structurally identical to enterobactin at the chelation site. Although not examined in this study, the ferrous iron transporter encoded by *sitABCD*, which is induced by *Shigella flexneri* during intracellular growth (41), may also contribute to UPEC iron acquisition during invasive UTI.

Our findings are generally consistent with previous studies examining the role of individual UPEC iron receptors in UTI. Our results indicated that in strain CFT073 heme receptors ChuA and Hma and aerobactin receptor IutA contributed most significantly to UTI. Similarly, *chuA*, *hma*, and *iutA* single mutants were previously shown to be outcompeted 15-, 38-, and 125-fold, respectively, by wild-type CFT073 (15, 49). In contrast, mutants lacking the enterobactin or salmochelin receptor *iha* or *iroN*, which contributed only slightly to UPEC colonization in our studies, were outcompeted only ~3-fold during cochallenge with the wild type (22, 43). This is the first report, however, to establish a role for yersiniabactin during UTI, as it was previously shown to be necessary for extraintestinal pathogenic *E. coli* virulence in a murine lethality model (44) and UPEC biofilm formation (16).

Although aerobactin, yersiniabactin, and heme receptor mutants were most often outcompeted by coinoculated mutants, the relative competitive fitness of each iron receptor mutant depended, to some extent, on the other mutants included in the mixed infection. We speculate that this may be due to the relative fitness of the coinoculated mutants, as well as inherent variability in the experimental model. For example, the $\Delta iutA$ mutant had reduced fitness in the bladder when competed against the relatively fit $\Delta fhuE$ and $\Delta fhuA$ fungal siderophore receptor mutants (Fig. 3A) but not when competed against the

likely less fit $\Delta iroN$, Δiha $\Delta fepA$, and Δhma $\Delta chuA$ mutants (Fig. 4B). While these differences indeed complicate our results, the use of independent replicates, as well as multiple combinations of mutants, with generally consistent results yields confidence in our findings.

Receptor cross-specificity, especially among the catecholate receptors, and the presence of undefined receptors in UPEC strains CFT073 and 536 complicate our findings. For example, while IroN is the predominant receptor for salmochelin, low uptake of this siderophore was also observed for Cir and FepA of *Salmonella* (19). Conversely, both FepA and IroN were demonstrated to transport enterobactin in *Salmonella* (37), and FepA, IroN, Cir, and Fiu all transport the enterobactin breakdown product DBS (17, 36). This overlapping substrate specificity likely contributed to the redundancy we observed among the catecholate receptors and may have diminished the relative importance of the individual receptors tested here. Consequently, while the relative role of salmochelin, for which a single receptor (IroN) exists, can be assessed by our studies, the relative role of enterobactin requires additional investigation. Future studies directly examining the relative role of catecholate siderophores in general will allow dissection of these redundancies as they relate to UPEC pathogenesis.

Additionally, several iron-responsive putative TonB-dependent receptors for which substrates have yet to be identified, including IreA and FitA, have been described in UPEC strains (34, 42). It is possible, and perhaps likely, that these receptors transport one of the siderophores examined here, thus adding complexity to an already complex system. These undefined receptors are expected to contribute to iron acquisition by UPEC during UTI, and future studies examining them within the context of siderophore and heme uptake will allow construction of a more complete hierarchy of UPEC iron uptake *in vivo*.

The entire length of the urinary tract is iron limiting, but the dramatic anatomical differences from urethra to kidneys likely offer substantial differences in iron source availability. Our results suggest that not only do certain iron acquisition systems appear more important than others for UPEC virulence but differences in the importance of these systems in specific urinary tract organs exist. Heme uptake, for example, was important consistently for kidney colonization and only rarely for bladder infection. In contrast, IutA-mediated aerobactin utilization was occasionally, but not always, found to contribute more significantly to bladder colonization than kidney infection. Variations in substrate availability or siderophore activity at disparate locations in the urinary tract may have contributed to these observed differences.

While the diverse iron acquisition systems of UPEC indeed provide redundancy for an essential biological function, our data establish that specific loci are more critical than others, often at distinct anatomical sites, indicating that a degree of specificity exists. Further delineation of these interactions will have implications for our overall understanding of UPEC virulence and pathogen evolution, as well as for the development of vaccines and therapeutics that target iron acquisition systems.

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